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Effect of long-term hyperosmolality on the Na^+/H^+ exchanger isoform NHE-3 in LLC-PK_1 cells

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Effect of long-term hyperosmolality on the Na^+/H^+ exchanger isoform NHE-3 in LLC-PK_1 cells. The effects of long-term exposure to hyperosmotic medium on the Na^+/H^+ exchanger isoform NHE-3 were examined in cultured renal epithelial cells (LLC-PK_1). LLC-PK_1 cells were grown to confluence in control medium (310 mOsm/kg H_2O) and then either switched to a hyperosmotic medium (510 mOsm/kg H_2O; addition of NaCl or mannitol) or maintained in the control medium for 48 hours. The Na^+/H^+ exchanger activity was then assessed in isosmotic solutions by measurement of amiloride-sensitive acid-stimulated $^{22}Na^+$ influx or Na^+-dependent acid extrusion. Acid-stimulated $^{22}Na^+$ influx was decreased significantly in cells incubated in hyperosmotic medium (10.5 ± 0.9 nmol/mg protein, control vs. 5.8 ± 0.6, hyperosmotic; $P < 0.01$). Incubation in hyperosmotic medium also decreased the initial rate of Na^+-dependent acid extrusion by ~~60% over the intracellular pH range 6.9 to 7.3. Intracellular buffering power did not differ in the control and hyperosmotic groups. The Na^+/H^+ exchanger isoform NHE-3 mRNA and protein, assessed by Northern hybridization and immunoblot analysis, respectively, were unchanged in LLC-PK_1 cells incubated in hyperosmotic medium compared with controls, suggesting post-translational regulation by high osmolality. These results demonstrate that long-term exposure to hyperosmotic medium causes an adaptive decrease in Na^+/H^+ exchange (NHE-3) activity in LLC-PK_1 cells, and that this effect is unlikely to involve antiporter gene regulation or a change in protein abundance.

Plasma membrane Na^+/H^+ exchange is a ubiquitous transporter in mammalian cells that plays a role in a variety of cell functions, including intracellular pH (pH_i) regulation, cell volume homeostasis, cell growth, and epithelial sodium reabsorption [1–5]. Recent molecular cloning studies have identified five isoforms of the Na^+/H^+ exchanger multigene family, designated NHE-1 through NHE-5 [6–11]. A major regulatory property of Na^+/H^+ exchange is its activation by hyperosmolality, a mechanism important for the control of cell volume. In many cell types, acute osmotic shrinkage causes parallel activation of Na^+/H^+ and Cl^-/HCO_3^- exchangers, resulting in net uptake of NaCl and H_2O that returns cell volume toward its original value [12–14]. Recent studies indicate, however, that Na^+/H^+ exchanger isoforms exhibit differential responses to short-term hyperosmolality: NHE-1 and NHE-2 activities are increased whereas NHE-3 activity is decreased [13–17]. In contrast, little is known about the regulation of Na^+/H^+ exchangers by long-term hyperosmolality. Chronic exposure of cultured inner medullary collecting duct (mIMCD-3) cells to hyperosmotic medium increased Na^+/H^+ exchange activity in association with an increase in NHE-2 mRNA and a decrease in NHE-1 mRNA [18]. In a more recent report, exposure of cultured vascular smooth muscle (VSM) cells to long-term hyperosmolality increased NHE-1 activity but had no effect on NHE-1 mRNA [19]. These findings indicate that NHE-1 and NHE-2 are stimulated by chronic as well as acute hyperosmolality, but that the molecular mechanisms involved in the chronic regulation may depend on the exchanger isoform studied and/or on the specific cell type in which the isoform is expressed.

The Na^+/H^+ exchanger isoform NHE-3 has been localized in the kidney to the apical membrane of proximal tubules and thick ascending limbs [20, 21]. This exchanger mediates transcellular reabsorption of NaCl and $NaHCO_3$ in the proximal tubule and reabsorption of $NaHCO_3$ in the thick ascending limb [21–24]. Recent studies indicate that short-term hyperosmolality inhibits both apical membrane Na^+/H^+ exchange activity and net HCO_3^- absorption in the rat medullary thick ascending limb [16, 23]. Acute exposure to high osmolality also inhibits NHE-3 activity in LLC-PK_1 and OK renal epithelial cell lines [15]. Thus, as noted above, the functional response of NHE-3 to acute hyperosmolality is opposite to that of NHE-1 and NHE-2, which are stimulated by acute hyperosmolality. The response of NHE-3 to long-term osmolality is not understood. Thus, it is unclear whether the unusual response of NHE-3 to acute osmotic stress persists with chronic hyperosmotic exposure. Accordingly, in the present study we examined the effects of long-term hyperosmolality on NHE-3 in the LLC-PK_1 cell line. The results show that long-term hyperosmolality causes a decrease in Na^+/H^+ exchange activity that persists when the cells are returned to isosmotic conditions. This adaptation is not associated with a change in NHE-3 mRNA or protein abundance, suggesting that it occurs independently of NHE-3 gene regulation and may involve post-translational mechanisms. The effect of chronic high osmolality to inhibit NHE-3 activity in renal tubule cells may contribute to the natriuresis and impaired urinary acidification observed in patients with diabetes mellitus, advanced renal failure, or methanol or ethylene glycol toxicity, conditions typically associated with increased plasma osmolality.

Key words: NHE-3, proximal tubule, hypertonicity, antiporter gene regulation, transporters.

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METHODS

Cell culture

LLC-PK_1 cells were grown in a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 50 mg/ml gentamycin. The cells were grown and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, LLC-PK_1 cells were grown to confluence on 13-mm plastic tissue culture dishes, glass coverslips, or 150-mm plates. They were then either switched to hyperosmotic medium (510 mOsm/kg H_2O) or maintained in the above control medium (310 mOsm/kg H_2O) for 48 hours. Medium osmolality was increased by the addition of either NaCl or mannitol.

Measurement of Na^+/H^+ exchange activity

$^{22}Na^+$ influx. The LLC-PK_1 cells were grown to confluence on 24-well plastic plates and then incubated in control or hyperosmotic medium for 24 or 48 hours. Na^+/H^+ exchange activity was assayed by measurement of $^{22}Na^+$ influx as previously described [15, 18, 19]. In brief, the cells were washed three times with a Na^+-free buffer consisting of 140 mM N-methyl-D-glucammonium (NMDG) Cl, 4 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4 (solution A). The cells were then incubated for 10 minutes in the same solution in which 30 mM $NMDG^+$ was replaced with 30 mM NH_4^+. The ammonium-containing solution was then replaced with solution A that contained 2 mM $^{22}NaCl$. $^{22}Na^+$ uptake was stopped after four minutes using four rapid washes with ice-cold saline. Cell-associated radioactivity was extracted with 1 ml of 1 N sodium hydroxide and quantified by scintillation counting. Protein concentration was determined by the bicinchoninic acid (BCA) method according to the manufacturer's protocol (Pierce, Rockford, IL, USA). Under the conditions of our experiments, virtually all ($\geq$ 97%) of acid-stimulated $^{22}Na^+$ uptake is mediated by Na^+/H^+ exchange [15].

Intracellular pH. LLC-PK_1 cells were grown on glass coverslips to confluence and then incubated in control or hyperosmotic medium for 48 hours. The monolayers were then transferred to a laminar-flow bath chamber on the stage of an inverted microscope and bathed at 37°C in solution A. The bath flow rate was 15 to 20 ml/min, which permitted complete exchange of the bathing solution within 600 ms. Intracellular pH was measured using the fluorescent probe 2′,7′-biscarboxyethyl-5(and-6)carboxyfluorescein (BCECF) [25, 26]. A diaphragm was placed over a 0.3 mm × 0.3 mm area of the confluent monolayer, background fluorescence was measured, and the cells were loaded with dye to ×20 background by bathing in 20 μM of the acetoxymethyl ester of BCECF. After washing, intracellular dye was excited alternately at 500 nm and 440 nm wavelengths using a computer-controlled spectrofluorometer (SPEX Industries). Fluorescence emission was monitored at 530 nm to obtain fluorescence excitation ratios (F500/F440) [25, 26]. Calibration of intracellular dye was performed at the end of each experiment using high K^+-nigericin standards as previously described [16, 26]. The initial rate of change in pH_i (dpH_i/dt) was calculated from the initial slope of the pH_i tracing measured over the first four seconds following addition of 50 mM Na^+ to the bathing solution (Na^+ replaced $NMDG^+$). In some experiments, pH_i recovery following Na^+ addition was interrupted at various points along the recovery curve by Na^+ replacement plus amiloride, as previously described [16]. This approach permits dpH_i/dt to be determined at more than one pH_i value in a given monolayer.

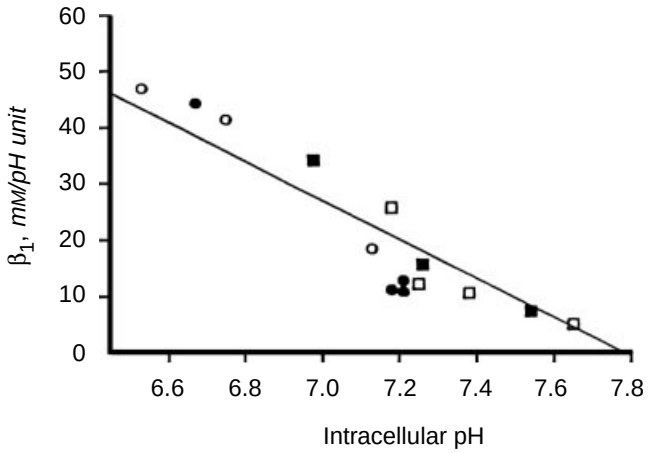

Fig. 1. Intrinsic buffering power (β_i) in LLC-PK_1 cells. Cells were incubated in control (filled symbols) or hyperosmotic (open symbols) medium for 48 hours. Hyperosmolality was produced by the addition of 100 mM NaCl. β_i values were determined in isosmotic solutions using ammonium (circles) or trimethylammonium (squares) as described in the **Methods** section. Data points are from single monolayers. The line is a linear regression over all data points (r = 0.91; y = 269.4–34.6x).

Intrinsic intracellular buffering power (β_i) was determined from the change in pH_i resulting from weak base addition [16, 27]. LLC-PK_1 monolayers were bathed initially in solution A. The addition of 2.5 mM ammonium (NH_4^+ replaced $NMDG^+$) caused pH_i to increase rapidly by ~~0.2 pH units, followed by a stable pH_i during the plateau phase. β_i was calculated as $d[NH_4^+]_i/dpH_i$, where dpH_i is the change in pH_i following ammonium addition and $d[NH_4^+]_i$ is the change in intracellular NH_4^+ concentration, calculated from its pKa (9.05 at 37°C) and pH_i, assuming that NH_3 concentration is equal in intracellular and extracellular fluids at steady state [16, 27]. To confirm the β_i values, additional measurements were obtained by adding 2.5 mM trimethylamine to monolayers bathed with (in mM) 50 NMDG gluconate, 100 K gluconate, 7 Ca gluconate, 1.5 Mg hydroxide, 5 HEPES, and 1 amiloride (pH 7.0 or 7.5), conditions used previously to measure β_i in thick ascending limbs [16]. As shown in Figure 1, β_i decreased linearly over the pH_i range 6.6 to 7.6, ranging from 48 mM/pH unit at pH_i 6.6 to 6 mM/pH unit at pH_i 7.6. β_i was unaffected by the growth conditions of the cells. Thus, the initial rates of Na^+-dependent cell alkalinization (dpH_i/dt) were used to compare Na^+/H^+ exchange activity in cells incubated in control and hyperosmotic medium (detailed in the **Results** section).

Isolation of total and poly(A)$^+$ RNA

Total cellular RNA was extracted from confluent LLC-PK_1 cells in multiple 100-mm dishes by the method of Chomczynski and Sacchi [28]. Cells were scraped from the dishes and homogenized in 10 to 12 volumes of 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Protein was extracted with Phenol:Chloroform (1:1) and RNA was precipitated using isopropanol. Poly(A)$^+$ RNA was prepared using Oligo dT cellulose spin columns (5′ Prime-3′ Prime, Inc., Boulder, CO, USA). Total and poly(A)$^+$ RNA were quantified by spectrophotometry.

Northern hybridization

Poly(A)$^+$ RNA samples, 20 mg/lane, were loaded and fractionated on 1% agarose-formaldehyde gels and transferred to nylon membranes [29]. The membranes were prehybridized for 15 hours at 42°C with 5× Denhardt's solution, 5 × SSC, 50% formamide, 0.5% SDS, and 0.5 mg/ml sheared salmon sperm DNA. The membranes were then hybridized overnight in the same solution that in addition contained 20 to 30 × 10^6 cpm ^{32}P-DNA probe for either NHE-3 or β-actin. The cDNAs were labeled with ^{32}P-deoxynucleotides using the Random Primed DNA labeling kit (5′ Prime-3′ Prime, Inc.). The membranes were washed in 2 × SSC, 0.5% SDS solution for 30 minutes at room temperature, 30 minutes at 50°C, and 45 minutes at 55°C. The membranes were then exposed to Kodak X-OMAT film at −70°C with intensifying screens for 72 to 120 hours. A rat NHE-3 cDNA fragment (*Pst*I-*Pst*I fragment, nucleotides 1153-2434) was used for Northern hybridization.

SDS-PAGE and immunoblot analysis

Microsomal membrane proteins (200 μg/lane) were isolated from LLC-PK$_1$ cells as described [15], solubilized and subjected to a vertical slab SDS-PAGE according to the Laemmli protocol [30]. Proteins were electrophoretically transferred to a nitrocellulose membrane at 200 mA for 15 hours. Nitrocellulose membranes were blocked in 3% BSA in phosphate buffered saline (PBST) and then incubated for two hours with 10 μl of NHE-3 immune serum diluted at 1:400 in PBST. This antibody is highly specific and does not cross react with other NHE isoforms [15, 31]. The excess antibody was washed with PBST and the antigen-antibody complex was treated with alkaline phosphatase-conjugated goat anti-rabbit IgG diluted at 1:1000 in PBST. The nitrocellulose membranes were developed with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) dissolved in 70% N,N,-dimethylformamide (DMF). Western blots were scanned using Image-1/MetaMorph Program.

Materials

^{22}Na, ^{32}P(dCTP) and ^{32}P(ATP) were purchased from New England Nuclear (Boston, MA, USA). Amiloride, dimethylamiloride (DMA), nigericin and nitrocellulose filters were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BCECF was obtained from Molecular Probes, Inc. (Eugene, OR, USA). 96-well microtiter plates were purchased from Becton-Dickson Labware (Oxnard, CA, USA). Oligo dT cellulose spin columns and Random-Primed DNA labeling kits were purchased from 5′Prime-3′Prime, Inc. NHE-3 cDNA was a generous gift from G. Shull (University of Cincinnati, Cincinnati, OH, USA).

Data analysis

The data are expressed as means ± SE. Statistical analysis was performed using unpaired *t*-test or ANOVA with Newman-Keuls' multiple range test. $P < 0.05$ was considered statistically significant.

RESULTS

Na^+/H^+ exchange expression in LLC-PK$_1$ cells

Recent studies using immunoblot analysis demonstrated that LLC-PK$_1$ cells express the Na^+/H^+ exchanger isoform NHE-3 on their apical membranes [15]. In the present study, two additional series of experiments were performed to confirm that LLC-PK$_1$ cells grown on non-permeable supports express NHE-3. In the first series, LLC-PK$_1$ cells were grown to confluence on plastic dishes and the sensitivity of $^{22}Na^+$ influx to amiloride analogs was examined. The acid-stimulated $^{22}Na^+$ influx rate was measured in the presence of two concentrations of amiloride (5 μM and 50 μM) and two concentrations of DMA (1 μM and 5 μM). Table 1 shows that $^{22}Na^+$ influx was relatively resistant to inhibition by amiloride and DMA (IC_{50} was approximately 50 μM for amiloride and 5 μM for DMA). These results are consistent with the functional expression of NHE-3, an isoform relatively resistant to inhibition by amiloride and its more potent analogs [4, 32, 33].[1] In a second series of experiments, we tested whether NHE-3 mRNA is expressed in the LLC-PK$_1$ cells. Poly(A)$^+$ RNA was isolated from confluent cultures grown on 150-mm dishes and expression of NHE-3 mRNA was assessed by Northern analysis. As shown in Figure 2, a 6.5 kb transcript was labeled following hybridization with NHE-3 cDNA, indicating expression of NHE-3 mRNA in the LLC-PK$_1$ cells.

Table 1. Inhibition of Na^+/H^+ exchange by amiloride and dimethylamiloride (DMA) in LLC-PK$_1$ cells

	Amiloride 5 μM	Amiloride 50 μM	DMA 1 μM	DMA 5 μM
Na^+ influx *% of control*	88 ± 6	51 ± 5[a]	92 ± 5	56 ± 4[a]

Acid-stimulated $^{22}Na^+$ influx was measured in the absence and presence of amiloride or DMA at the concentrations indicated. Values are means ± SE for 3 experiments in each condition.

[a] Amiloride or DMA significantly different from control.

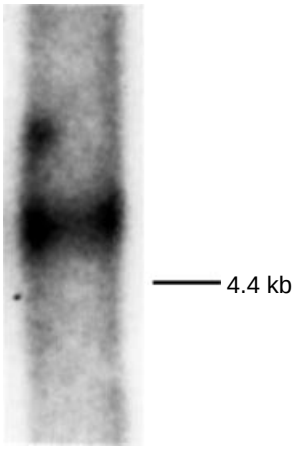

Fig. 2. A representative Northern blot of poly(A)$^+$ RNA from LLC-PK$_1$ cells for NHE3. Twenty micrograms of poly(A)$^+$ RNA were size fractionated and probed with rat NHE-3 cDNA fragment *Pst*I-*Pst*I (nucleotides 1153-2434).

[1] In addition to NHE-3, LLC-PK$_1$ cells also express the amiloride-sensitive exchanger isoform NHE-1 [34]. We have shown previously in LLC-PK$_1$ cells grown to confluence on non-permeable supports that concentrations of DMA (0.5 μM) that inhibit NHE-1 at a Na^+ concentration of 2 mM [4] reduce acid-stimulated $^{22}Na^+$ influx by less than 5% [15]. Thus, under the conditions of our experiments, virtually all of Na^+/H^+ exchange activity is attributable to the amiloride-resistant NHE-3 isoform.

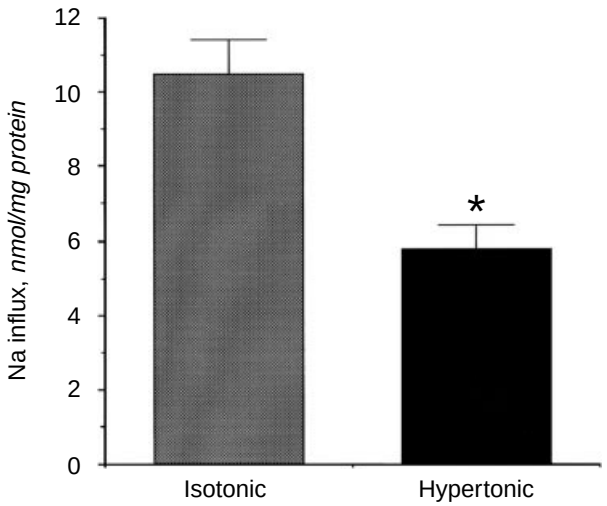

Fig. 3. Effect of long-term hyperosmolality on DMA-sensitive $^{22}Na^+$ influx in LLC-PK$_1$ cells. Cells were grown in 24-well plastic plates and exposed to control (310 mOsm/kg H_2O) or hypertonic (510 mOsm/kg H_2O; added NaCl) medium for 48 hours. Thereafter, the media were aspirated, the cells were acidified, and ^{22}Na influx was assayed at four minutes in isosmotic solution in the absence and presence of 0.1 mM DMA (**Methods**). Values are means ± SE for four separate experiments performed in quadruplicate. *Hypertonic significantly different from control.

Long-term hyperosmolality inhibits Na^+/H^+ exchange activity

Previous studies have shown that acute increases in osmolality inhibit NHE-3 activity in LLC-PK$_1$ cells [15] and in rat medullary thick ascending limbs [16]. In the present study, we examined the effects of long-term hyperosmolality on Na^+/H^+ exchange activity in LLC-PK$_1$. Cells were grown to confluence on 24-well plates and then incubated in either control (310 mOsm/kg H_2O) or hyperosmotic (510 mOsm/kg H_2O; added NaCl) medium for 48 hours. The media were then removed and both groups of cells were incubated in control medium for 45 minutes. Thereafter, the cells were acid loaded and assayed for Na^+/H^+ exchange activity using $^{22}Na^+$ influx as described in the **Methods** section. As shown in Figure 3, acid-stimulated, DMA-sensitive $^{22}Na^+$ uptake was decreased significantly in LLC-PK$_1$ cells incubated for 48 hours in hyperosmotic medium ($P < 0.01$). To determine whether the difference in $^{22}Na^+$ influx may have been the indirect result of a difference in pH_i, we measured pH_i under the same conditions used in the $^{22}Na^+$ experiments. In six separate experiments, steady-state pH_i was 7.46 ± 0.05 in controls and 7.39 ± 0.06 in the high osmolality group ($P > 0.05$). In addition, the minimum pH_i achieved following removal of NH_4^+ was 6.43 ± 0.04 in controls and 6.37 ± 0.05 in high osmolality ($P > 0.05$), indicating that the extent of cell acidification at the time of measurement of $^{22}Na^+$ influx was similar in the two groups. These results indicate that the decrease in amiloride-sensitive $^{22}Na^+$ uptake in cells incubated in hyperosmotic medium represents an adaptive inhibition of the exchanger, and is not the indirect result of a difference in pH_i under the assay conditions.

To further identify the influence of long-term hyperosmolality on Na^+/H^+ exchange activity, we measured rates of Na^+-dependent acid extrusion. Cells incubated for 48 hours in control (310 mOsm/kg H_2O) or hyperosmotic medium (510 mOsm/kg H_2O) were removed from the medium and then equilibrated in Na^+-free solution (solution A) in the microfluorometer bath chamber until a stable pH_i value was obtained (~ 20 min). Na^+/H^+ exchange activity was assessed from the initial rate of increase in pH_i (dpH_i/dt) following rapid addition of 50 mM Na^+ to the bath solution (**Methods**), and the results are shown in Figure 4. In both groups of cells, dpH_i/dt decreased with increasing pH_i over the range of 6.8 to 7.6 (Fig. 4A). Forty-eight hours of incubation in the hyperosmotic medium decreased the rate of Na^+-dependent acid extrusion at all pH_i values studied. Hyperosmotic incubation decreased dpH_i/dt by 63% at pH_i 6.95 and by 65% at pH_i 7.25 (Fig. 4B)[2]. To confirm that the Na^+-dependent acid extrusion was mediated via Na^+/H^+ exchange, pH_i recovery was monitored following addition of 50 mM Na^+ to the bath in the presence of 1 mM amiloride. At pH_i 6.94 ± 0.03, dpH_i/dt was 0.02 ± 0.01 ($N = 4$) in control cells and 0.03 ± 0.01 ($N = 4$) in cells incubated in hyperosmotic medium. Thus, the Na^+-dependent pH_i recovery was inhibited nearly completely by amiloride.

In the above experiments, hypertonicity was achieved by addition of 100 mM NaCl to the media. To determine whether the suppression of Na^{+/H^+} exchange activity is a general response to hypertoncity or is unique to increased [NaCl], LLC-PK$_1$ cells were grown to confluence in 24-well plates and exposed to a hypertonic medium (510 mOsm/kg H_2O) produced by the addition of either 100 mM NaCl or 200 mM mannitol. The media were then removed after 48 hours. As shown in Figure 5, acid-stimulated, DMA-sensitive $^{22}Na^+$ uptake was decreased significantly in both hypertonic media compared to control ($P < 0.01$ and $P < 0.03$ for hypertonicity induced by NaCl and mannitol, respectively, vs. control, $N = 4$).

To examine the tonicity dependence of Na^+/H^+ exchange inhibition by hypertonic media, cells were incubated for 48 hours in either control medium or hypertonic media produced by the addition of various concentrations of NaCl (410, 510 or 610 mOsm/kg H_2O). The media were then removed, cells were incubated in control medium for 45 minutes, and the Na^+/H^+ exchanger activity was assayed in a manner similar to Figure 3. As shown in Figure 6, acid-stimulated, DMA-sensitive $^{22}Na^+$ uptake was decreased in an osmotically-dependent manner, with 610 mOsm/kg H_2O media causing the most and the 410 mOsm/kg H_2O media causing the least degree of inhibition.

To examine the time course of Na^+/H^+ exchange inhibition by hyperosmolality, cells were incubated for 24 and 48 hours in either control (310 mOsm/kg H_2O) or hypertonic medium (510 mOsm/kg H_2O; added NaCl). The media were then removed and the Na^+/H^+ exchanger activity was assayed in a manner similar to Figure 3. As shown in Figure 7, acid-stimulated, DMA-sensitive $^{22}Na^+$ uptake was decreased as early as 24 hours after exposure to

[2] In theory, the decrease in dpH_i/dt in the hyperosmotic cells could be the result of an increase in cell volume rather than a decrease in Na^+/H^+ exchanger activity. This possibility is very unlikely for several reasons. First, the volume of the hyperosmotic cells would have to be more than twice that of the control cells to account for the decrease in dpH_i/dt. No such volume difference was visible in the monolayers. Second, a change in volume would alter β_i by affecting the concentrations of cell buffers; however, β_i was similar in control and hyperosmotic cells (Fig. 1). Third, the influence of a change in volume is offset by a change in β_i of inverse proportion, which tends to maintain constant the product $\beta_i \cdot V$ in control and hyperosmotic conditions [16]. Fourth, incubation in hyperosmotic medium reduced DMA-sensitive $^{22}Na^+$ uptake, an independent measure of Na^+/H^+ exchange activity that is not influenced directly by cell volume.

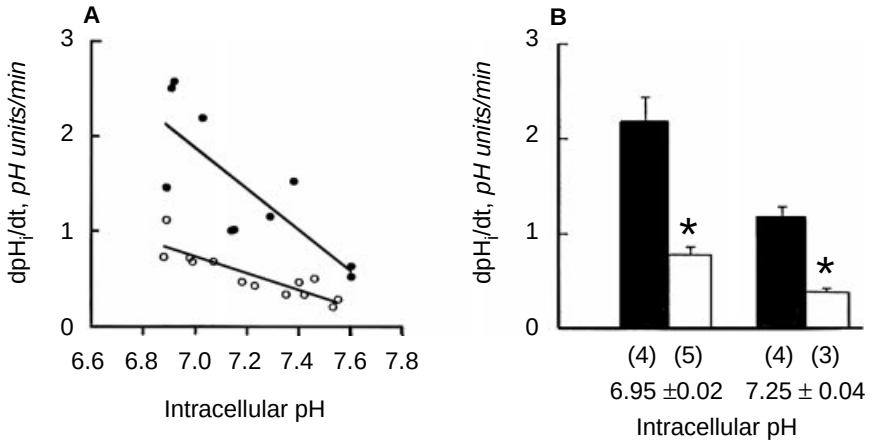

Fig. 4. Effects of long-term hyperosmolality on Na^+-dependent acid extrusion in LLC-PK_1 cells. Cells were incubated for 48 hours in control or hyperosmotic medium and then initial rates of Na^+-dependent acid extrusion (dpH_i/dt) were measured as described in the **Methods** section. (*A*) dpH_i/dt as a function of pH_i. Data points were obtained from 6 control (●) and 5 hyperosmotic (○) monolayers. Lines are linear regressions over all data points (control, $r = 0.79$, $y = 16.9–2.2x$; hyperosmolar, $r = 0.87$, $y = 6.9–0.9x$). (*B*) Mean dpH_i/dt at two values of pH_i. Symbols are: (■) control; (□) hyperosmotic. Bars are means ± SE for data points grouped over intervals of 0.3 pH unit. Numbers in parentheses are numbers of monolayers studied for each condition. *$P <$ 0.01, hyperosm versus control.

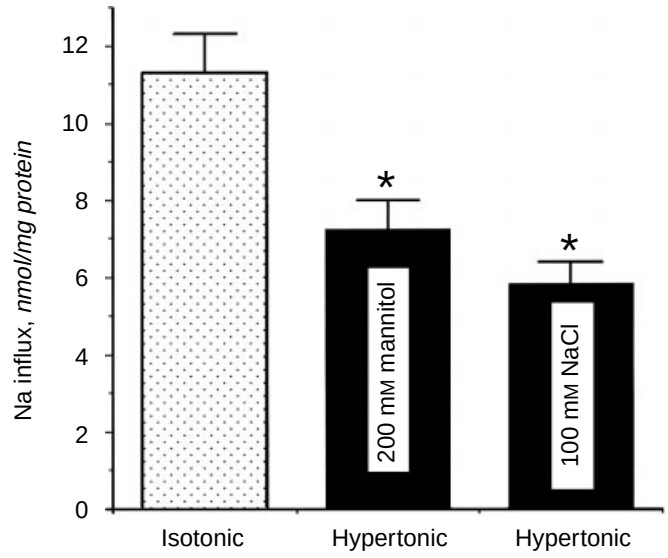

Fig. 5. Effect of mannitol or NaCl as osmotic solute on DMA-sensitive $^{22}Na^+$ influx in LLC-PK_1 cells. Cells were grown in 24-well plastic plates and exposed to control (310 mOsm/kg H_2O) or hypertonic medium (200 mM mannitol or 100 mM NaCl added to the control medium) for 48 hours. Thereafter, the media were aspirated, the cells were acidified, and ^{22}Na influx was assayed at four minutes in isosmotic solution in the absence and presence of 0.1 mM DMA. Values are means ± SE for four separate experiments performed in quadruplicate. *Hypertonic significantly different from control.

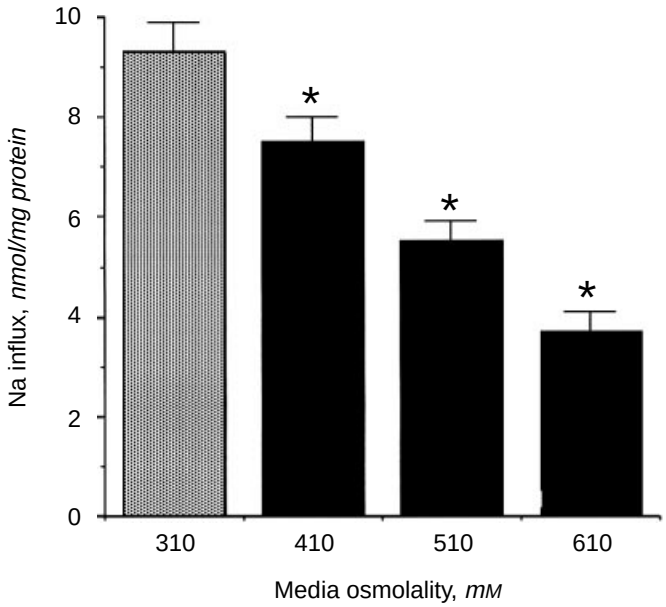

Fig. 6. Effect of increasing tonicity on DMA-sensitive $^{22}Na^+$ influx in LLC-PK_1 cells. Cells were grown in 24-well plastic plates and exposed to control (310 mOsm/kg H_2O) or various hyperosmotic media (410, 510 or 610 mOsm/kg H_2O; added NaCl) medium for 48 hours. Thereafter, the media were aspirated, the cells were acidified, and ^{22}Na influx was assayed at four minutes in isosmotic solution in the absence or presence of 0.1 mM DMA (**Methods**). Values are means ± SE for four separate experiments performed in quadruplicate. *Hyperosmotic significantly different from the previous group.

hypertonicity ($P < 0.05$ for 24 hr and $P < 0.01$ for 48 hr of hypertonicity vs. control, $N = 4$). The magnitude of inhibition was less severe at 24 hours versus 48 hours ($P < 0.05$, $N = 4$), indicating the time dependence of Na^+/H^+ exchange activity by hypertonicity.

Long-term hyperosmolality does not decrease NHE-3 mRNA or protein abundance

The results in Figures 3 to 5 indicate that long-term exposure to hyperosmotic medium inhibits Na^+/H^+ exchange activity in LLC-PK_1 cells. To determine whether this effect may involve regulation at the transcription level, we examined the effect of elevated osmolality on steady-state levels of Na^+/H^+ exchanger (NHE-3) mRNA. LLC-PK_1 cells were grown on 100 mm dishes and exposed to either control or hyperosmotic medium for 48 hours. $Poly(A)^+$ RNA from each group was size fractionated, transferred to a nylon membrane, and probed with radiolabeled NHE-3 cDNA. As shown in Figure 8, the mRNA levels for NHE-3 and the constitutive control (β-actin) were not affected by hyperosmolality. The Northern blots were performed on $poly(A)^+$ RNA samples that were pooled from ten 100-mm plates of LLC-PK_1 cells incubated in control or hyperosmotic medium. Densitometric scanning of blots from three separate experiments showed no significant difference between the control and high osmolality groups (97 ± 5% hyperosmolality vs. 100% control; $P > 0.05$).

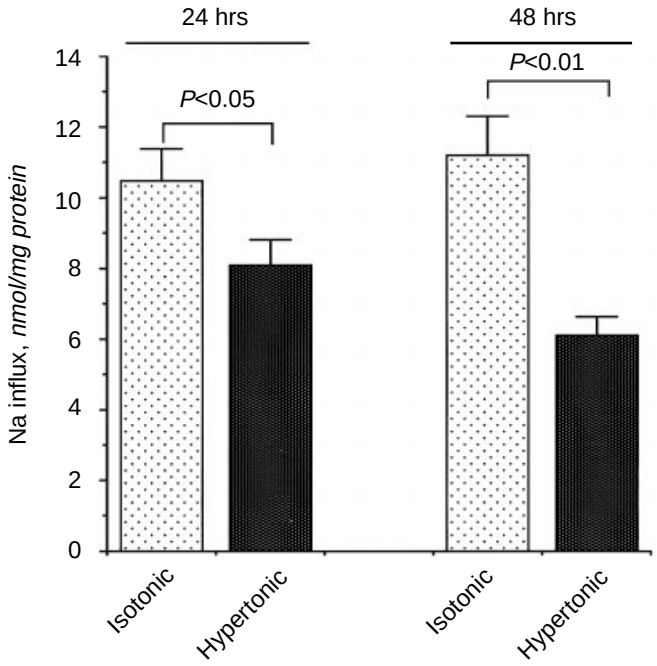

Fig. 7. Time course of inhibition of DMA-sensitive $^{22}Na^+$ influx by hypertonicity in LLC-PK$_1$ cells. Cells were grown in 24-well plastic plates and exposed to control (310 mOsm/kg H_2O) or hypertonic (510 mOsm/kg H_2O; added 100 mM NaCl) medium for 24 or 48 hours. Thereafter, the media were aspirated, the cells were acidified, and the DMA-sensitive ^{22}Na influx was assayed at four minutes in isosmotic solution. Values are means ± SE for four separate experiments performed in quadruplicate.

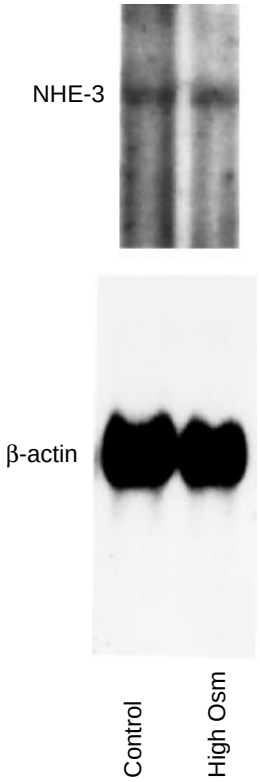

Fig. 8. Northern blot analysis of NHE-3 mRNA. LLC-PK$_1$ cells were grown in 100-mm dishes and incubated in control or hyperosmotic medium for 48 hours. Poly(A)$^+$ RNA was extracted from both groups, size fractionated, and probed for NHE3 mRNA as described in Figure 2. β-actin mRNA levels were measured as constitutive controls.

To determine if long-term hypertoncity affects NHE-3 protein abundance, microsomal membrane proteins were isolated from LLC-PK$_1$ cells that were grown on 100-mm dishes and incubated in either control (310 mOsm/kg H_2O) or hyperosmotic medium (510 mOsm/kg H_2O; added NaCl) for 48 hours. Membrane proteins (200 μg/lane) were resolved by SDS-PAGE and probed with an NHE-3 immune serum. As shown in Figure 9, the abundance of NHE-3 protein did not differ in the hypertonic group (right lane) compared to control (left lane). The abundance of NHE-3 in the hypertonic cells was 94% ± 7 compared to control ($P > 0.05$, $N = 3$).

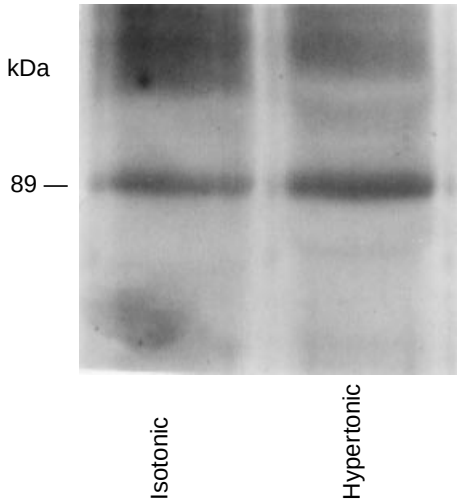

Fig. 9. NHE-3 immunoblot analysis. Representative Western blots showing NHE-3 protein abundance in LLC-PK$_1$ cells incubated in control (left lane) or hypertonic (right lane) medium for 48 hours. Hyperosmolality was produced by the addition of 100 mM NaCl. The NHE-3 molecular weight was ~94 kDa.

DISCUSSION

The present study examined the effects of long-term hyperosmolality on the activity of the Na^+/H^+ exchanger isoform NHE-3 in LLC-PK$_1$ cells. The results demonstrate that 48 hours of incubation in hyperosmotic medium causes a decrease in Na^+/H^+ exchange activity that persists when the cells are removed from the hyperosmotic medium. The decrease in exchanger activity under the conditions of the assay occurs independent of pH_i and is not associated with a change in the NHE-3 mRNA level or protein abundance. Thus, the inhibition of NHE-3 by chronic hyperosmolality is an adaptive process that occurs by mechanisms other than a change in NHE-3 gene transcription or translation.

Previous studies have demonstrated that NHE isoforms respond differently to acute increases in extracellular osmolality. NHE-1, which is expressed ubiquitously in plasma membranes of nonepithelial cells and basolateral membranes of epithelia, is stimulated by acute hyperosmolality, a response that is important for the maintenance of steady-state cell volume [12–15, 17]. NHE-2 is expressed at low levels in the kidney and has been localized to the basolateral membrane of cultured inner medullary

collecting duct cells (mIMCD-3) [9, 10, 18]. Similar to NHE-1, NHE-2 is stimulated by acute hyperosmolality, suggesting that it may be involved in the short-term control of cell volume [17, 18]. In contrast, a different response is observed for NHE-3, the amiloride-resistant isoform present in the apical membrane of intestinal epithelial cells and renal proximal tubule and thick ascending limb cells [4, 20–23]. Short-term hyperosmolality inhibits NHE-3 in the rat medullary thick ascending limb [16], in the renal epithelial cell lines opossum kidney (OK) and LLC-PK_1 [15], and when the exchanger is expressed in an antiporter-deficient cell line [17]. Inhibition by acute hyperosmolality thus appears to be an intrinsic property of the NHE-3 isoform, independent of the cell type in which it is expressed. The results of the present study extend these observations by demonstrating in LLC-PK_1 cells that NHE-3 also is inhibited by chronic hyperosmolality. In contrast, chronic high osmolality has been shown to stimulate NHE-1 and NHE-2 activities in other systems [18, 19]. Thus, although the results of the chronic studies must be interpreted with caution since the isoforms were studied in different cell types, it appears that the differential effects of hyperosmolality to stimulate NHE-1 and NHE-2 and inhibit NHE-3 are manifest not only as acute regulation but also as chronic adaptations in response to prolonged hyperosmotic exposure.

The results of our experiments further indicate that inhibition of NHE-3 activity by hypertonicity in LLC-PK_1 cells is independent of the osmotic solute. An increase in tonicity by the addition of either NaCl or mannitol for 48 hours caused suppression of Na^+/H^+ exchange activity (Fig. 5), indicating that the inhibition of NHE-3 reflects a general response to hypertonicity and is not unique to NaCl. Our results further indicate that NHE-3 inhibition could be detected as early as 24 hours after exposure to hyperosmotic media (Fig. 7).

Although the mechanism by which chronic hyperosmolality inhibits NHE-3 was not identified in our experiments, it is possible to comment on some of the factors that may influence Na^+/H^+ exchange activity. Our results show that, under the conditions of the assay, the effect of long-term hyperosmolality to decrease Na^+/H^+ exchage activity was independent of pH_i (Fig. 4). Furthermore, the inhibition of Na^+/H^+ exchange is unlikely to have been due to a difference in intracellular Na^+ activity, since $^{22}Na^+$ uptake and Na^+-dependent acid extrusion rates were measured in Na^+-depleted cells. These findings indicate that the decrease in the NHE-3 transport rate was not the result of a decrease in the net driving force for the exchanger,[3] which suggests that hyperosmolality inhibits the exchanger by altering either the activity of the individual transporters (that is, the turnover number) and/or the number of copies of the transporter in the cell membrane. With respect to the latter possibility, the results in Figures 8 and 9 show that long-term exposure to hyperosmotic medium did not decrease the NHE-3 mRNA or protein abundance. Consequently, transcriptional regulation of the exchanger is unlikely to be involved in the decrease in Na^+/H^+ exchange activity. Furthermore, since NHE-3 accounts for virtually all of Na^+/H^+ exchange activity under the conditions of our experiments, the decrease in the exchange rate cannot be explained by a decrease in the transcription or activity of another exchanger isoform. Thus, when taken together, our results suggest that long-term hyperosmolality inhibits NHE-3 activity through posttranslational mechanisms, such as regulated trafficking, changes in exchanger phosphorylation, or synthesis or activation of a protein that regulates NHE-3 activity.

Although our experiments do not provide evidence for a role for transcriptional regulation of NHE-3 by hyperosmolality in LLC-PK_1 cells, chronic regulation of NHE-3 activity through changes in NHE-3 gene transcription has been reported in other systems. Studies in OKP cells have shown that chronic incubation in acid or dexamethasone causes an increase in NHE-3 activity that is associated wth elevated NHE-3 mRNA levels via increased transcription [35, 36]. This NHE-3 gene regulation likely contributes to the effects of chronic acidosis and glucocorticoids to increase apical membrane Na^+/H^+ exchange activity and HCO_3^- absorption in the renal proximal tubule [5, 36, 37]. In addition, in a recent preliminary study, incubation of OKP cells for 48 hours in medium made hyperosmotic by the addition of glucose or mannitol induced an increase in NHE-3 activity that was associated with increases in NHE-3 protein abundance and mRNA levels [38]. It is presently unclear why chronic hyperosmolality inhibits NHE-3 activity with no change in mRNA level in LLC-PK_1 cells, while stimulating NHE-3 activity and mRNA level in OKP cells. Factors that may contribute to the differing responses include differences in the experimental procedures or cell-specific regulation of signaling pathways or accessory proteins that influence NHE-3 activity. Further evidence that chronic regulation of Na^+/H^+ exchange activity by hyperosmolality can occur independent of changes in exchanger mRNA level was obtained in recent studies of NHE-1 in vascular smooth muscle cells [19]. Taken together, these studies indicate that long-term regulation of Na^+/H^+ exchange by high osmolality is a complex and cell-specific process that may involve changes in exchanger gene transcription as well as posttranslational mechanisms. Further work is needed to identify the posttranslational factors that may be involved in chronic osmotic regulation of Na^+/H^+ exchange activity.

Recently we demonstrated that long-term high osmolality increases Na^+/H^+ exchange (NHE-1) activity in vascular smooth muscle cells via chronic activation of protein kinase C (PKC) [19]. Activation of PKC has been shown acutely to stimulate NHE-1 and inhibit NHE-3 when these exchangers are expressed in antiporter-deficient cell lines [3, 4, 33, 39]. These findings raise the possibility that PKC could mediate chronic inhibition of NHE-3 by hyperosmolality in LLC-PK_1 cells. Protein kinase C activity, assessed by phosphorylation of a PKC-specific substrate [19, 40, 41], was found to be elevated in LLC-PK_1 cells incubated in hyperosmotic medium (29 ± 1.8 pmol phosphorylation/mg/min, control vs. 36 ± 2.1, hyperosmotic, $N = 4$; $P < 0.05$). However, addition of the PKC inhibitor H7 did not prevent inhibition of NHE-3 (M. Soleimani, unpublished data). Thus, although long-term hyperosmolality induces chronic activation of PKC in LLC-PK_1 cells, this pathway is unlikely to be responsible for the adaptive inhibition of NHE-3 activity. Alternatively, acute inhibition of apical Na^+/H^+ exchange (NHE-3) by hyperosmolality in the rat medullary thick ascending limb is mediated via a protein tyrosine kinase-dependent signaling pathway that functions independent of regulation by PKC or cAMP [23]. Furthermore,

[3] Intracellular pH and Na^+ activity were not monitored during the 24 or 48 hour period of exposure of cells to control and hyperosmotic media. Thus, our experiments do not rule out the possibility that a change in one or both of these parameters during that time may have contibuted to the adaptation in the Na^+/H^+ exchanger.

tyrosine kinase pathways involving c-src have been shown to participate in chronic regulation of NHE-3 by acid in cultured proximal tubule cells [42]. These findings suggest that activation of tyrosine kinase pathways may be responsible for chronic inhibition of NHE-3 activity by hyperosmolality. Further work is needed to identify signaling pathways involving tyrosine phosphorylation that are regulated by hyperosmotic stress in renal tubules.

In summary, the results of this study demonstrate that long-term exposure to hyperosmotic medium causes an adaptive decrease in Na^+/H^+ exchange (NHE-3) activity in LLC-PK_1 cells. This adaptation is not associated with a change in NHE-3 mRNA or protein abundance. Thus, the inhibition likely is mediated by post-translational mechanisms, independent of NHE-3 gene regulation. In view of the key role of NHE-3 in mediating renal NaCl and $NaHCO_3$ reabsorption, we suggest that chronic inhibition of this exchanger may contribute to natriuresis and impaired urinary acidification in conditions associated with long-term high osmolality, such as advanced renal failure.

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APPENDIX

Abbreviations used in this article are: BCA, bicinchoninic acid; BCECF, 2′,7′-biscarboxyethyl-5(and -6)carboxyfluorescein; BCIP, bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; DMA, dimethylamiloride; DMEM, Dulbecco's modified Eagle's medium; DMF, N,N,-dimethylformamide; dpH_i/dt, initial rate of change in intracellular pH; mIMCD-3, basolateral membrane of the cultured inner medullary collecting duct; NBT, nitro blue tetrazolium; NHE-3, Na^+/H^+ exchange; NMDG, N-methyl-D-glucammonium; OK, opossum kidney; PBST, phosphate buffered saline with BSA; pH_i, intracellular pH; PKC, protein kinase C; VSM, vascular smooth muscle cells.

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